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# Transformation of an interleukin 3-dependent hematopoietic cell line by the chronic myelogenous leukemia-specific P210$^{bcr/abl}$ protein

**(Abelson murine leukemia virus/Philadelphia chromosome/tyrosine kinase)**

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**ABSTRACT** **The P210$^{bcr/abl}$ protein is associated with virtually every case of human chronic myelogenous leukemia. Unlike the related P160$^{gag/v-abl}$ oncogene product of Abelson murine leukemia virus, P210$^{bcr/abl}$ does not transform NIH 3T3 fibroblasts. To assess whether P210$^{bcr/abl}$ might transform hematopoietic cell types, retroviral constructs encoding P210$^{bcr/abl}$ were used to infect the bone marrow-derived interleukin 3-dependent Ba/F3 cell line. As for P160$^{gag/v-abl}$, cell lines expressing P210$^{bcr/abl}$ were growth factor independent and tumorigenic in nude mice. No evidence for autocrine production of interleukin 3 by factor-independent cell lines was found. These experiments establish that P210$^{bcr/abl}$ can transform hematopoietic cell types to tumorigenicity.**

The P210$^{bcr/abl}$ protein derives from a hybrid gene created by the chromosomal translocation that generates the Philadelphia chromosome, a cytogenetic abnormality which characterizes human chronic myelogenous leukemia (CML) cells (1). The CML-specific P210 protein shares structural and enzymatic properties with the v-abl protein of the Abelson murine leukemia virus (A-MuLV). The gene for P210 and v-*abl* arose by substitution of the c-*abl** sequence encoding the N-terminal region with *bcr* (2) and helper-virus-derived *gag* sequences (3), respectively. Both proteins exhibit elevated tyrosine-specific protein kinase activity (4). The v-abl protein is responsible for the induction of acute lymphosarcomas in susceptible murine hosts infected with A-MuLV (5). The role of P210 in the etiology of human CML remains to be defined.

The v-abl protein can transform a variety of cell types. It efficiently transforms NIH 3T3 fibroblasts *in vitro* (6) and pre-B-lymphoid cells both *in vitro* and *in vivo* (7) and is able to relieve the growth-factor dependence of several hematopoietic cell types, including T-cell lines dependent on interleukin 2 (8), and various interleukin 3 (IL-3)-dependent lymphoid and myeloid cell lines (9–13). Unlike v-abl, P210 does not transform NIH 3T3 fibroblasts (14). In this study, we demonstrate that P210 resembles v-abl in its ability to transform the IL-3-dependent hematopoietic cell line Ba/F3 (15) to factor independence and tumorigenicity. These experiments demonstrate the oncogenic potential of P210$^{bcr/abl}$.

## MATERIALS AND METHODS

**Plasmid Constructs, Viral Stocks, and Cell Culture Conditions.** A 4.7-kilobase (kb) fragment containing coding sequence for the full-length P160 isolate of v-*abl* (16) was cloned into the *Bam*HI cloning site of the pWE vector (B. Guild and R. C. Mulligan, Whitehead Institute) by using *Bam*HI linkers. The resulting size for the pWEgab construct was 8.7 kb from the 5′ long terminal repeat (LTR) to the 3′ LTR. The full-length P210$^{bcr/abl}$ cDNA 172/215 (17) was cloned into pWE by using *Bcl* I linkers. The resulting LTR–LTR size for the pWE210 construct was 11 kb. Viral stocks were generated by the following methods. Replication-competent retroviral complexes were made by cotransfection of the constructs with helper Moloney murine leukemia virus DNA (pZAP, ref. 18) onto NIH 3T3 cells. Alternatively, helper-free retroviral stocks were made by transfecting the constructs onto the packaging line $\psi$-2 (19) and infecting tunicamycin-treated $\psi$-2 cells with a transient viral harvest from the transfected $\psi$-2 cells. Retroviral supernatants from G418-resistant producer populations were harvested after 18 hr and passed through a 0.45-$\mu$m filter. Viral supernatants were titered for G418 resistance by infection of NIH 3T3 fibroblasts. All retroviral producer cell lines had comparable titers in the range of $10^5$ G418-resistant colony-forming units/ml.

Ba/F3 is a murine bone marrow-derived cell line dependent on IL-3 for viability and proliferation (15). Ba/F3 cells are classified as early cells of the lymphoblastoid lineage by virtue of their low-level expression of the B-cell-specific B220 antigen (unpublished data) and the germ-line configuration of their immunoglobulin loci. Ba/F3 cells were maintained in standard RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum and 10% (vol/vol) conditioned medium from the WEHI-3B cell line (as a source of IL-3, ref. 20). Ba/F3 cells are able to grow in medium supplemented with fetal calf serum and purified recombinant IL-3 alone (unpublished data). Ba/F3 cells not only fail to proliferate but also rapidly die in the absence of an exogenous source of IL-3. Numerous experiments with the Ba/F3 cell line conducted in this laboratory have failed to generate any spontaneous IL-3-independent clones. Ba/F3 cells ($2 \times 10^6$ cells) were infected by incubation with 2 ml of viral supernatant and Polybrene at 8 $\mu$g/ml for 2 hr or by a 48-hr cocultivation with helper-free packaging lines in the presence of Polybrene at 2 $\mu$g/ml. Infected Ba/F3 cells were maintained in the presence of WEHI-3B conditioned medium for 48 hr and then selected for retroviral infection in G418 at 2 mg/ml. G418-resistant populations were washed twice with isotonic phosphate-buffered saline (PBS) before plating in medium lacking a source of IL-3 to select for IL-3-independent growth.

**Assay of Cell Proliferation.** Cell proliferation was assessed by an adaptation of the tetrazolium dye reduction assay of Mosmann (21). Relevant Ba/F3 cell lines were washed twice with PBS and resuspended at $5 \times 10^5$ cells per ml in RPMI medium containing 5% (vol/vol) fetal calf serum. Cells were

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Abbreviations: CML, chronic myelogenous leukemia; A-MuLV, Abelson murine leukemia virus; IL-3, interleukin 3; LTR, long terminal repeat.

*In this report, lowercase italic gene symbols will be used for all genes, regardless of species of origin.

dispensed in triplicate into 96-well plates ($2.5 \times 10^4$ cells per well). Test supernatants were added in appropriate dilution to the plates, which were incubated for 24 hr at 37°C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) was added to each well, and the cells were further incubated for 5 hr. The absorbance was measured on a Dynatech model MR580 plate reader at a wavelength of 570 nm. A neutralizing polyclonal anti-IL-3 IgG was a gift from P. Vassalli (University of Geneva), and a control polyclonal IgG specific for the epidermal growth factor receptor was a gift of Y. Yarden (Whitehead Institute). IgG was preincubated with conditioned medium for 1 hr prior to addition to the proliferation assay.

**Nucleic Acid and Protein Analysis.** High molecular weight genomic DNA was isolated from relevant cell lines, digested with the enzyme *Xba* I, electrophoresed through a 0.7% agarose gel, transferred to a nylon membrane, and probed with a fragment of the G418-resistance (Neo) gene labeled to high specific activity ($>10^9$ cpm/$\mu$g). Hybridization and wash conditions were of high stringency. Immunoprecipitation analysis of abl proteins was as described (22).

**Assay of Tumorigenicity in Nude Mice.** Cells for assay of tumorigenicity were washed with serum-free medium and resuspended in Hanks' balanced saline solution. About $2 \times 10^6$ cells were injected subcutaneously into young (<12 week old) nude mice (BALB/c-AnNCr-nu) from the National Cancer Institute. Mice received 500 rads (1 rad = 0.001 Gy) of $\gamma$-irradiation 24 hr prior to cell challenge. Mice were observed for 2–3 months for signs of palpable or visible tumor at the site of injection. Tumorigenic cell lines gave rise to a visible pea-sized mass with a short latency after injection which was nonregressing and malignant. Nontumorigenic cell lines showed no evidence of tumor for up to 3 months after injection.

## RESULTS

Constructs for expression of abl protein variants were made by using the pWE vector (Fig. 1). The pWE vector carries a dominant coselectable antibiotic-resistance marker expressed from the promoter element of the Moloney virus LTR. The cloned *abl* sequence is expressed from an internal promoter derived from the chicken $\beta$-actin gene. Titers of retroviral producer cell supernatants were determined by resistance of infected NIH 3T3 fibroblasts to the antibiotic G418 and were comparable for all constructs ($10^5$ colony-forming units/ml). IL-3-dependent Ba/F3 cells were infected with retroviral-producer cell culture supernatants and grown for 48 hr in the presence of WEHI-3B conditioned medium prior to selection (Fig. 2). Primary selection for viral infection was carried out in medium supplemented with both WEHI-3B conditioned medium and G418 at 2 mg/ml. For all infected cell lines, G418-resistant populations arose after 7–10 days of selection, suggesting that $\approx$0.1% of cells had been infected. To select for IL-3 independence, G418-resistant populations were washed extensively with PBS and cultured in medium lacking a source of IL-3. Cell populations infected with the pWE210 virus (encoding $P210^{bcr/abl}$) or the pWEgab virus (encoding $P160^{gag/v\text{-}abl}$) gave rise to populations of IL-3-independent cells after 5–10 days, suggesting that between 0.1% and 5% of cells survived selection. A G418-resistant population of cells infected with the pWE virus alone or uninfected cells did not survive selection in medium lacking a source of IL-3, demonstrating that *abl* sequences are required to generate IL-3 independence (Table 1).

Uninfected Ba/F3 cells proliferate maximally in the presence of 3–10% (vol/vol) conditioned medium from WEHI-3B cells, and their proliferation declines upon dilution of the conditioned medium (Fig. 3). The proliferation profile for Ba/F3 cells infected with the pWE virus alone is similar to that for the uninfected Ba/F3 cell line (Fig. 3*A*). Cells infected with viruses that encode $P210^{bcr/abl}$ or $P160^{gag/v\text{-}abl}$ and selected for growth in the absence of exogenous IL-3 proliferate in a factor-independent manner, without regard to the concentration of conditioned medium from WEHI-3B cells. Their growth rate is equivalent to the parental Ba/F3 cell line growing in medium supplemented with IL-3 (unpublished data). Conditioned medium from the IL-3-independent cell lines infected with either the pWE210 or pWEgab viruses will not support the proliferation of uninfected Ba/F3 cells. This suggests that the pWE210- and pWEgab-infected cells do not liberate a growth factor into the medium that can support their growth by autocrine stimulation. Analysis of total RNA from the IL-3-independent cell lines failed to detect expression of IL-3 mRNA, although it could readily be detected in WEHI-3B cells (unpublished data). Fig. 3*B* shows the results of experiments with a specific antibody to IL-3 capable of neutralizing its growth-promoting activity. IgG directed at an irrelevant antigen (the epidermal growth factor receptor) failed to alter the proliferation profile for uninfected Ba/F3 cells. IgG specific for IL-3 inhibited the proliferative activity of WEHI-3B conditioned medium for uninfected Ba/F3 cells. The IL-3-independent Ba/F3 cells infected with either the pWE210 or pWEgab viruses were assayed in medium lacking WEHI-3B conditioned medium in the presence of neutralizing anti-IL-3 IgG. The proliferation of the pWE210- and pWEgab-infected cells was unaffected by the presence of the neutralizing IgG.

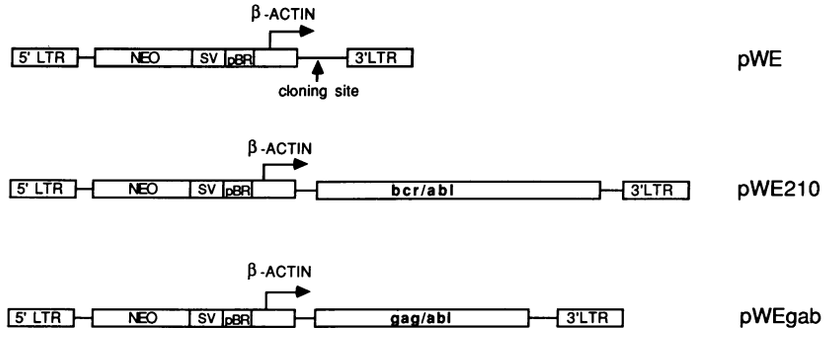


FIG. 1. Retroviral constructs. The pWE vector is a derivative of the Moloney murine leukemia virus (B. Guild and R. C. Mulligan, Whitehead Institute). The Neo gene, encoding resistance to the antibiotic G418, is expressed from the 5' LTR. An internal promoter from the chicken $\beta$-actin gene directs the expression of cloned cDNA inserted into the unique *Bam*HI cloning site. SV and pBR represent sequences for the simian virus 40 and pBR322 plasmid origins of replication. For pWE210, the cDNA for the full-length coding region of the $P210^{bcr/abl}$ protein (cDNA 172/215, ref. 14) was cloned into the pWE vector by using *Bcl* I linkers. For pWEgab, sequences encoding the $P160^{gag/v\text{-}abl}$ isolate of A-MuLV (16) were cloned into the pWE vector by using *Bam*HI linkers.

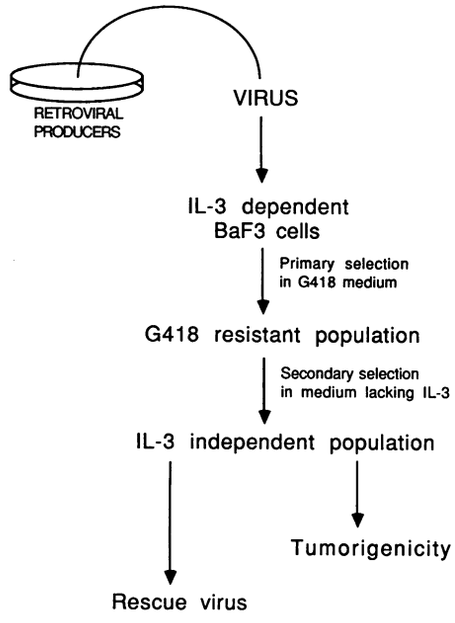


FIG. 2. Strategy for generation and analysis of IL-3-independent Ba/F3 cell lines. Retroviral producer cell lines were generated. Ba/F3 cells were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum and 10% (vol/vol) conditioned medium from WEHI-3B cells (a source of IL-3, ref. 20). Approximately $2 \times 10^6$ Ba/F3 cells were incubated with retroviral supernatant for 2 hr in the presence of Polybrene at 8 μg/ml. Alternatively, cells were infected by a 48-hr cocultivation with helper-free retroviral producer cell lines in the presence of Polybrene at 2 μg/ml. Infected cells were grown for 48 hr before primary selection for retroviral integration in medium containing G418 at 2 mg/ml. G418-resistant populations of cells were washed twice with PBS before secondary selection for IL-3 independence in medium lacking a source of IL-3. Virus was rescued from IL-3-independent cell lines by superinfection with helper Moloney virus.

Analysis of the proviral structure in the IL-3-independent cell lines showed the pWE210 and pWEgab viruses to be intact and unrearranged. Digestion of total genomic DNA with the restriction endonuclease *Xba* I, which cuts within the retroviral LTRs to expose the full-length provirus, demonstrated the expected fragment sizes for the relevant constructs (Fig. 4). The correct proviral structure was detected by either the G418-resistance (Neo) gene or c-abl probe sequences (unpublished data). The provirus in these cell lines was rescued by superinfection with Moloney helper virus. Virus rescued from either pWE210- or pWEgab-infected IL-3-independent cell lines passed the G418-resistance marker upon infection of NIH 3T3 cells, but only virus rescued from pWEgab-infected cell lines was able to transform NIH 3T3 cells (Table 1). The properties of the rescued virus match those of the original viral stock used to effect the IL-3-independent phenotype for Ba/F3 cells.

Cell extracts of relevant cell lines were immunoprecipitated with anti-abl antisera (23) and processed for the immune-complex kinase reaction with [$\gamma$-$^{32}$P]ATP. The precipitated proteins were displayed by NaDodSO$_4$/polyacrylamide gel electrophoresis and visualized by autoradiography (Fig. 5). Cell lines infected with the pWE210 and pWEgab viruses and selected in G418 expressed the P210$^{bcr/abl}$ and P160$^{gag/v\text{-}abl}$ proteins, respectively. IL-3-independent cell populations invariably expressed higher levels of abl protein, suggesting that IL-3 independence requires high-level expression of abl protein.

IL-3-independent cell lines expressing P210$^{bcr/abl}$ and P160$^{gag/v\text{-}abl}$ (either with helper virus or helper-free) were assayed for their tumorigenicity in nude mice. The parental Ba/F3 cells, or Ba/F3 cells infected with the pWE virus alone, did not form tumors upon subcutaneous challenge. The IL-3-independent, pWE210-infected, and pWEgab-infected cells readily formed tumors at the site of subcutaneous injection that were of short latency, nonregressing, and ultimately lethal to the host (Table 1).

## DISCUSSION

A common denominator in CML is the Philadelphia chromosome, a t(9;22) that is detectable in a high proportion (>90%) of patients (24). The Philadelphia translocation juxtaposes c-*abl* protooncogene sequences on chromosome 9 with a locus on chromosome 22 (*bcr*) that encodes a protein of unknown function (25). The ability to detect the molecular rearrangement of *bcr* and *abl* in a proportion of the CML patients for whom cytogenetic evidence of the Philadelphia chromosome is lacking (26) further strengthens the association of the aberrant chromosome with the disease. The product of the Philadelphia chromosome, the P210$^{bcr/abl}$ fusion protein, resembles the P160$^{gag/v\text{-}abl}$ oncogene product in enzymatic and structural properties. The c-*abl* sequences that are deleted in the formation of P210 and P160 v-abl share homology with nonreceptor tyrosine kinases (e.g., *src* and *fps*), phospholipase C, and the avian v-*crk* oncogene (27, 28). Simple deletion of this region within the c-*abl* type IV sequence is sufficient to activate the transforming potential of c-*abl* for fibroblasts and lymphoid cells (P. Jackson and D.B., unpublished results). The association of the Philadelphia

Table 1. Properties of infected Ba/F3 cells

| Virus | Protein | Growth of Ba/F3 cells in selective medium after viral infection: Primary, + IL-3/ + G418 | Growth of Ba/F3 cells in selective medium after viral infection: Secondary, − IL-3/ + G418 | Tumors in nude mice | Ability of conditioned medium to support Ba/F3 cell proliferation | Properties of rescued virus: Passes Neo gene | Properties of rescued virus: Transforms 3T3 cells |
|---|---|---|---|---|---|---|---|
| Mock | | − | | 0/8 | | | |
| pWE | | + | − | 0/7 | | | |
| pWEgab | P160$^{gag/v\text{-}abl}$ | + | + | 9/9 | − | + | + |
| pWE210 | P210$^{bcr/abl}$ | + | + | 12/12 | − | + | − |

For the capacity to establish Ba/F3 cell line under conditions of primary selection (G418 resistance) or secondary selection (IL-3 independence), + indicates establishment of continuously proliferating cell line and − indicates cell line will not grow under specified conditions. To establish whether infected cells could produce tumors in nude mice, the number of mice that developed large, nonregressing tumors at the site of subcutaneous injection of cells over the number of mice challenged is shown. To determine whether conditioned medium could support Ba/F3 cell proliferation, conditioned medium was harvested from helper-virus-free cell lines at high density and passed through a 0.45-μm filter. −, Cells could not proliferate. To establish properties of rescued virus, Ba/F3 cell lines were superinfected with helper Moloney murine leukemia virus. Rescued retroviral supernatant was passed through a 0.45-μm filter before infection of NIH 3T3 cells. Infected cells were assayed for passage of the G418-resistance (Neo) gene by selection in G418 at 1 mg/ml or were allowed to grow to confluence and examined for the appearance of foci of transformed cells. +, Virus has indicated property; −, virus lacks the property.

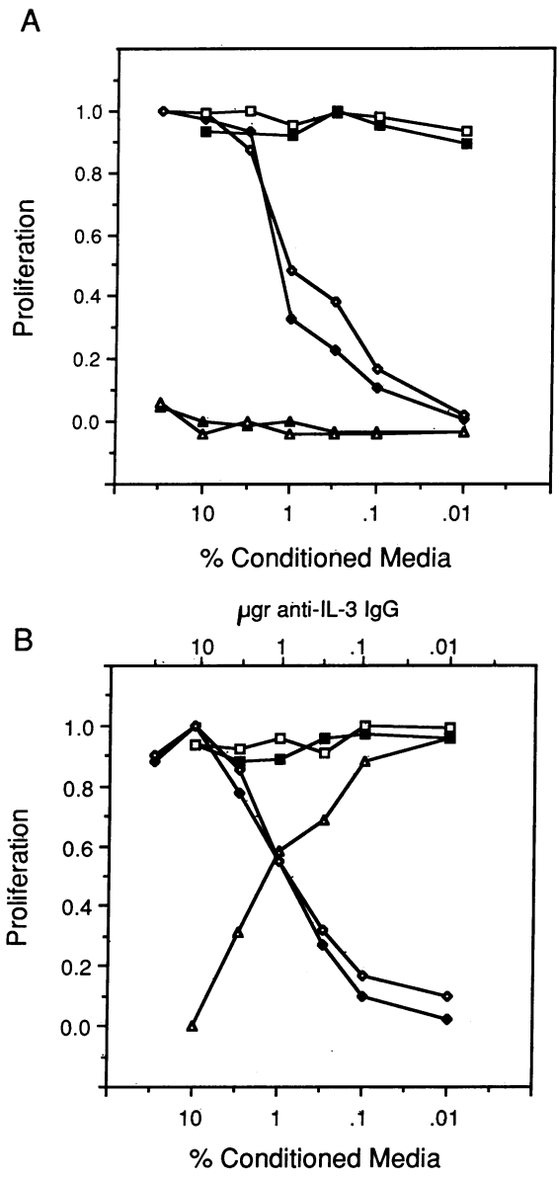


FIG. 3. Proliferation assay of Ba/F3 cell lines. Effect of dilution of conditioned medium on proliferation of various Ba/F3 cell lines. Conditioned medium is from the WEHI-3B cell line, a source of IL-3, unless otherwise noted. (*A*) ◆, Uninfected cells; ◇, pWE-infected cells; ■, pWEgab-infected cells; □, pWE210-infected cells; ▲, uninfected Ba/F3 cells plus conditioned medium from pWE210-infected Ba/F3 cells; △, uninfected Ba/F3 cells plus conditioned medium from pWEgab-infected Ba/F3 cells. (*B*) ◆, Uninfected cells; ◇, uninfected cells assayed in presence of 10 μg of control IgG (anti-epidermal growth factor receptor antibody); △, uninfected cells assayed in 3% (vol/vol) WEHI-3B conditioned medium to dilution of a neutralizing anti-IL-3 IgG; ■, pWEgab-infected cells assayed in medium lacking IL-3 to the indicated dilution of neutralizing anti-IL-3 IgG; □, pWE210-infected cells assayed in medium lacking IL-3 to the indicated dilution of neutralizing anti-IL-3 IgG. Anti-IL-3 IgG (10 μg) neutralized 100% of the proliferative activity of 3 μl of WEHI-3B conditioned medium for Ba/F3 cells. Proliferation measurements have been normalized by subtracting the optical density (570 nm) for nonproliferating cells from each assay point and dividing by the optical density of maximally proliferating cells.

chromosome with virtually every case of CML and the similarity of $P210^{bcr/abl}$ to the v-*abl* oncogene product of the Abelson murine leukemia virus strongly implicates P210 in the pathogenesis of CML.

A previous study demonstrated the growth-promoting

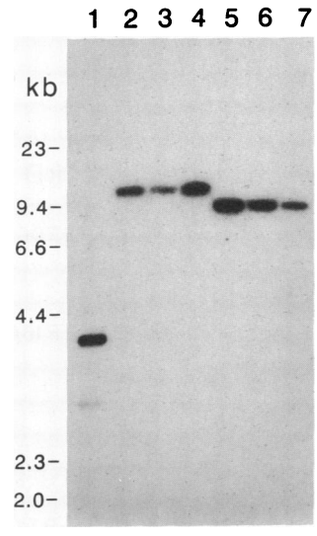


FIG. 4. Proviral structure in infected G418-resistant Ba/F3 cell lines. Genomic DNA (2 μg) isolated from relevant cell lines was digested with the enzyme *Xba* I to demonstrate full-length provirus. Hybridization with a probe derived from the G418-resistance (Neo) gene occurred under conditions of high stringency. Lanes: 1, pWE-infected Ba/F3 cells maintained in medium supplemented with IL-3; 2, pWE210-infected cells maintained in medium supplemented with IL-3 prior to selection for IL-3 independence; 3 and 4, pWE210-infected cell lines selected for IL-3-independent growth in medium lacking IL-3; 5, pWEgab-infected cells maintained in medium supplemented with IL-3; 6 and 7, pWEgab-infected cell lines selected for IL-3-independent growth.

effects of P210 on early cells of the B-lymphoid lineage in the Whitlock–Witte bone marrow culture system (29). Not all of the clonal lines stimulated by P210 in that system were tumorigenic. Reminiscent of disease progression from chronic to acute phase, some clones progressed to tumorigenicity upon passage in culture, suggesting that secondary events were necessary in acquiring the full-tumorigenic phenotype. Whereas that study involved infection of primary bone marrow, the Ba/F3 cell line used in these experiments has been adapted for continuous growth in culture and may be more permissive for transformation.

This study demonstrates that the gene for $P210^{bcr/abl}$ can function as a dominant oncogene. Like v-*abl*, it will transform the bone-marrow-derived IL-3-dependent Ba/F3 cell line to factor independence and tumorigenicity. P210 does not trigger the endogenous expression of IL-3 or other growth factors capable of stimulating Ba/F3 proliferation in an autocrine manner. The proliferation of the P210-transformed cells in the presence of a neutralizing anti-IL-3 antibody argues that the cells are not hyperresponsive to undetectable levels of IL-3 present in the growth medium. Rather, $P210^{bcr/abl}$ must itself provide the stimulus for Ba/F3 cell proliferation normally provided through the IL-3 signal transduction pathway.

These experiments establish the oncogenic potential of $P210^{bcr/abl}$ for the lymphoblastoid cell line Ba/F3. Preliminary results suggest that P210 will transform the IL-3-dependent mast cell line 32Dc13 to factor independence, thus demonstrating transformation for myeloid cell types (unpublished data). Unlike v-abl, P210 cannot transform NIH 3T3 fibroblasts unless it recombines with N-terminal gag sequences from the helper virus, which provides a myristoylation function critical for fibroblast transformation (14). Given that P210 will not transform fibroblasts, the requirements for transformation of adherent and nonadherent cell types by abl proteins appear to be distinct.

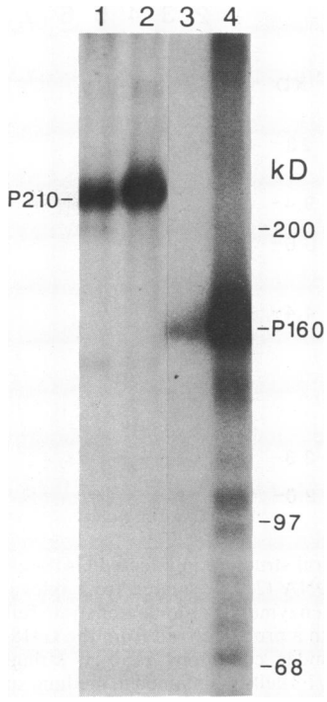


FIG. 5. Immunoprecipitation analysis of infected Ba/F3 cell lines. Cell extracts were incubated with anti-abl antisera (pEX4/5 mixture, ref. 23) and processed for *in vitro* immune-complex kinase reaction as described (22). Proteins were displayed by $NaDodSO_4$/polyacrylamide gel electrophoresis and visualized by autoradiography. Each lane was normalized for total protein content. Lanes: 1, pWE210-infected cells maintained in IL-3 prior to selection for IL-3 independence; 2, pWE210-infected cells selected for IL-3-independent growth; 3, pWEgab-infected cells maintained in IL-3 prior to selection for IL-3 independence; 4, pWEgab-infected cells selected for IL-3-independent growth.

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